

# The Calcium Dependence of Pigment Translocation in Freshwater Shrimp Red Ovarian Chromatophores

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**Abstract.** The roles of calcium in cell signaling consequent to chromatophorotropin action and as an activator of mechanochemical transport proteins responsible for pigment granule translocation were investigated in the red ovarian chromatosomes of the freshwater shrimp *Macrobrachium olfersii*. Chromatosomes were perfused with known concentrations of free  $\text{Ca}^{++}$  ( $10^{-3}$  to  $10^{-9}$  M) prepared in  $\text{Mg}^{++}$ -EGTA-buffered physiological saline after selectively permeabilizing with 25  $\mu\text{M}$  calcium ionophore A23187 or with  $10^{-8}$  M red pigment concentrating hormone (RPCH). The degree of pigment aggregation and the translocation velocity of the leading edges of the pigment mass were recorded in individual chromatosomes during aggregation induced by RPCH or A23187 and dispersion induced by low  $\text{Ca}^{++}$ . Aggregation is  $\text{Ca}^{++}$  dependent, showing a dual extracellular and intracellular requirement. After perfusion with reduced  $\text{Ca}^{++}$  ( $10^{-4}$  to  $10^{-9}$  M), RPCH triggers partial aggregation ( $\approx 65\%$ ), although the maximum translocation velocities ( $\approx 16.5$   $\mu\text{m}/\text{min}$ ) and velocity profiles are unaffected. After aggregation induced at or below  $10^{-5}$  M  $\text{Ca}^{++}$ , spontaneous pigment dispersion ensues, suggesting a  $\text{Ca}^{++}$  requirement for RPCH coupling to its receptor, or a concentration-dependent,  $\text{Ca}^{++}$ -induced  $\text{Ca}^{++}$ -release mechanism. The  $\text{Ca}^{++}$ -channel blockers  $\text{Mn}^{++}$  (5 mM) and verapamil (50  $\mu\text{M}$ ) have no effect on RPCH-triggered aggregation. An intracellular  $\text{Ca}^{++}$  requirement for aggregation was demonstrated in chromatosomes in which the  $\text{Ca}^{++}$  gradient across the cell membrane was dissipated with A23187. At free  $[\text{Ca}^{++}]$  above  $10^{-3}$  M, aggregation is complete; at  $10^{-4}$  M, aggregation is partial, followed by spontaneous dispersion; below  $10^{-5}$  M  $\text{Ca}^{++}$ , pigments do not aggregate but disperse slightly. Aggregation velocities diminish from  $11.6 \pm 1.2$

$\mu\text{m}/\text{min}$  at 5.5 mM  $\text{Ca}^{++}$  to  $7.4 \pm 1.3$   $\mu\text{m}/\text{min}$  at  $10^{-4}$  M  $\text{Ca}^{++}$ . Half-maximum aggregation occurs at  $3.2 \times 10^{-5}$  M  $\text{Ca}^{++}$  and half-maximum translocation velocity at  $4.8 \times 10^{-5}$  M  $\text{Ca}^{++}$ . Pigment redispersion after 5.5 mM  $\text{Ca}^{++}$ -A23187-induced aggregation is initiated by reducing extracellular  $\text{Ca}^{++}$ ; slight dispersion begins at  $10^{-7}$  M, complete dispersion being attained at  $10^{-9}$  M  $\text{Ca}^{++}$ . Dispersion velocities increase from  $0.6 \pm 0.2$  to  $3.1 \pm 0.5$   $\mu\text{m}/\text{min}$ . Half-maximum dispersion occurs at  $7.6 \times 10^{-9}$  M  $\text{Ca}^{++}$  and half-maximum translocation velocity at  $2.9 \times 10^{-9}$  M  $\text{Ca}^{++}$ . These data reveal an extracellular and an intracellular  $\text{Ca}^{++}$  requirement for RPCH action, and demonstrate that the centripetal or centrifugal direction of pigment movement, the translocation velocity, and the degree of pigment aggregation or dispersion attained are calcium-dependent properties of the granule translocation apparatus.

## Introduction

Chromatic adaptation in the decapod Crustacea is brought about by the differential translocation of colored pigment granules contained within specialized, multicellular effectors known as chromatosomes (McNamara, 1981). The mechanisms responsible for the centripetal and centrifugal movement of the granules within the constituent chromatophores are regulated by small peptide hormones of neurosecretory origin, often specific and antagonistic for each pigment cell type, but generally termed pigment concentrating (PCH) and pigment dispersing (PDH) chromatophorotropins (Josefsson, 1983; Rao and Fingerma, 1983; Fingerma, 1985).

The signal transduction pathways and intracellular enzymatic cascades activated by these peptides are poorly known in the Crustacea, although they are fairly well established in vertebrate groups like the teleosts, amphibians,

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and reptiles, which also exhibit color changes regulated by (neuro)endocrine and neural mechanisms (reviews in Novales, 1983; Nery and Castrucci, 1997; Tuma and Gelfand, 1999). Both intracellular free  $Ca^{++}$  concentrations and cyclic nucleotides have been implicated in the second messenger systems that respectively activate the pigment aggregating and dispersing mechanisms in the Crustacea (Fingerman, 1969; Rao and Fingerman, 1983; Nery *et al.*, 1997, 1998). However, it is difficult to establish generic regulatory mechanisms because the two principal groups studied, the brachyuran crabs and the caridean shrimps, differ considerably in their pigmentary responses to the same effector agents. To illustrate, chromatosome pigments spontaneously disperse in shrimp epidermal preparations *in vitro* (Fingerman *et al.*, 1975; Lambert and Fingerman, 1979; McNamara and Taylor, 1987; Tuma *et al.*, 1993), but they aggregate in crab preparations, in the absence of agonists (Lambert and Fingerman, 1976; Kulkarni and Fingerman, 1986). Intracellular  $Ca^{++}$ , increased by the calcium ionophore A23187 and calcium-containing salines, causes pigment aggregation in shrimps (Lambert and Fingerman, 1979; Britto *et al.*, 1990; McNamara and Ribeiro, 1999) but dispersion in crabs (Quackenbush, 1981). In shrimps, cyclic AMP disperses red chromatophore pigments (Fingerman, 1969; Nery *et al.*, 1998), but in crabs it is either without effect or also disperses chromatophore pigments (Quackenbush, 1981; Rao and Fingerman, 1983). However, cyclic GMP also disperses black and white but not red chromatophore pigments in crabs (Quackenbush, 1981) and has no effect on shrimp red chromatophores (Nery *et al.*, 1998). Clearly, unequivocal signal transduction pathways that modulate the molecular motors responsible for pigment aggregation and dispersion are yet to be established in the Crustacea as a whole.

Alterations in intracellular calcium concentrations appear to play a major role in the signaling transduction pathway or effector mechanism regulating the mechanochemical protein motors responsible for pigment translocation in caridean chromatophores. Red pigment concentrating hormone (RPCH) induces the aggregation of dispersed pigments, but its action is inhibited or impaired in  $Ca^{++}$ -free media (Lambert and Fingerman, 1979; McNamara and Taylor, 1987; Britto *et al.*, 1996) and in the presence of verapamil, an L-type  $Ca^{++}$  channel blocker (Britto *et al.*, 1996). In contrast, dispersion of A23187- or RPCH-aggregated pigments can be induced in  $Ca^{++}$ -free saline (Lambert and Fingerman, 1979; Britto *et al.*, 1990; McNamara and Ribeiro, 1999). However, unlike red chromatophores in which pigment aggregation is induced by  $Ca^{++}$  (Luby-Phelps and Porter, 1982; McNamara and Ribeiro, 1999; Kotz and McNiven, 1994), in crustacean chromatophores the origin and role of  $Ca^{++}$  in pigment aggregation is unclear, and no estimates

are available of changes in intracellular  $Ca^{++}$  concentration during pigment movements.

Using red ovarian chromatosomes from *Macrobrachium olfersii*, a freshwater caridean shrimp, the present study investigates the origin and calcium-dependent nature of pigment aggregation and dispersion induced by RPCH and A23187. The intracellular free  $[Ca^{++}]$  associated with activation of the respective granule translocation mechanisms is also estimated with the aid of a  $Mg^{++}$ -EGTA buffer system.

## Materials and Methods

Immature, female freshwater shrimp, *Macrobrachium olfersii*, presenting small, translucent ovaries, were collected from the Paúba River in São Paulo State, Brazil, and maintained under a natural photoperiod in about 100 l of river water (salinity < 0.5‰, temperature  $\approx 23^\circ C$ ) in 250-l tanks; they were fed celery, beetroot, carrot, and minced beef or chicken.

The ovarian chromatosome preparation employed has been described in detail previously (McNamara and Ribeiro, 1999). Briefly, after dissection, the red chromatosomes were gravity perfused (0.7 ml/min) in an acrylic microperfusion chamber (150  $\mu$ l volume) and observed at 320 $\times$  by reflected and transmitted light using a Wild M10 stereoscopic microscope coupled to a Sony DXC-151A CCD video camera and Trinitron monitor.

An ocular graticule was used to quantify the diameter of the pigment mass in chromatosomes of 220–240  $\mu$ m diameter at 2-min intervals. The data were converted to percent maximum dispersion, and the translocation velocity (in micrometers per minute) of the leading edge of the pigment mass was calculated, for each interval, according to McNamara and Ribeiro (1999).

Perfusion salines were prepared based on ionic data from McNamara *et al.* (1990) for *M. olfersii*. The control saline contained (in millimoles):  $Na^+$  177,  $K^+$  5,  $Ca^{++}$  5.5, and  $Mg^{++}$  1 as chlorides ( $\approx 195$ ).

Salines of specific low  $Ca^{++}$  concentration were prepared using a  $Mg^{++}$ -10 mM EGTA buffer system. For RPCH- and A23187-induced pigment aggregation, the respective  $[Ca^{++}]$  and  $[Mg^{++}]$  necessary to furnish final free  $Ca^{++}$  concentrations of  $10^{-3}$  to  $10^{-9}$  M, while holding free  $[Mg^{++}]$  at 1.03 mM, were calculated using the MCalc computer program based on Fabiato (1988). For pigment dispersion in previously A23187-perfused preparations, the  $[Ca^{++}]$  and  $[Mg^{++}]$  given by Luby-Phelps and Porter (1982) were used to provide final free  $Ca^{++}$  concentrations of  $10^{-7}$ ,  $10^{-8}$ , and  $10^{-9}$  M at a final free  $[Mg^{++}]$  of 1.3 mM. In the  $Ca^{++}$ -free saline (residual  $[Ca^{++}] \approx 7 \times 10^{-11}$  M),  $Ca^{++}$  was substituted by 27.5 mM choline chloride, and 2 mM EDTA was added.

All salines contained 0.5% DMSO (dimethyl sulfoxide; Sigma, MO), 2.5 mM Na<sub>2</sub>HCO<sub>3</sub>, and 2 mM glucose (osmolality prior to DMSO, 354 ± 0.7 mOsm/kg H<sub>2</sub>O) and were adjusted to pH 7.4. Calcium ionophore A23187 (free acid, Sigma, MO), dissolved in DMSO, was used at a final concentration of 25 μM. Red pigment concentrating hormone (Peninsula Laboratories Inc., CA), dissolved in distilled water or 10% DMSO, was used at a final concentration of either 10 or 30 nM. Verapamil hydrochloride and MnCl<sub>2</sub> (Sigma, MO) were dissolved in DMSO or distilled water and used at final concentrations of 50 μM (Hille, 1992) and 5 mM (Zhang *et al.*, 1997), respectively. All experiments were performed at room temperature (≈23°C). Under these conditions, the chromosome preparation is functional for at least 3–4 h, and up to five successive cycles of aggregation and dispersion can be induced using A23187 and Ca<sup>++</sup>-free saline, respectively.

Each experiment was repeated seven times, using measurements from a single chromosome in each preparation. Since most data were not normally distributed, the treatment effect was evaluated using the Kruskal-Wallis one-way, nonparametric ANOVA, followed by Dunn's test to locate significantly different groups. Correlations between pigment translocation velocities and degree of aggregation or dispersion were evaluated employing Pearson's product moment correlation test (SigmaStat 2.03, SPSS Inc., CA). Concentration-effect curves and the 50% response values were obtained using the dose-response logistic curve-fitting function of SlideWrite 5.0 Plus (Advanced Graphics Software, Inc., CA). All tests were performed with a significance level of  $P = 0.05$ .

## Results

### Calcium dependence of pigment aggregation

**Red pigment concentrating hormone.** On perfusion with 10 nM RPCH in control (5.5 mM Ca<sup>++</sup>) saline, the dispersed pigments aggregate rapidly and completely within 24 min (Fig. 1A1). Translocation velocity attains a customary peak of 17.0 ± 2.9 μm/min ( $n = 11$ ) with a small right-hand shoulder, followed by a short plateau of 1.8 ± 0.3 μm/min, gradually declining thereafter (Fig. 1B1). RPCH washout induces immediate dispersion, reaching about 90% within about 40 min (Fig. 1A1). Dispersion velocity attains a maximum of 5.6 ± 2.5 μm/min (Fig. 1B1).

Perfusion with 10 nM RPCH and the various Mg<sup>++</sup>-EGTA-buffered Ca<sup>++</sup>-salines reveals a hormone effect notably dependent on extracellular Ca<sup>++</sup>. In 10<sup>-3</sup> M Ca<sup>++</sup>, the degree of aggregation and the velocity profile (Fig. 1A2, 1B2) are similar to those in control saline (5.5 mM Ca<sup>++</sup>, Fig. 1A1, 1B1). However, in salines containing reduced Ca<sup>++</sup>, maximum aggregation varies from 90% (10<sup>-4</sup> M, Fig. 1A3) to 63% (10<sup>-5</sup> M, Fig. 1A4), 67% (10<sup>-6</sup> M, Fig.

1A5) and 60% (10<sup>-9</sup> M, Fig. 1A6), requiring about 13 min. However, the peak translocation velocities (≈16.5 μm/min) are unaffected by [Ca<sup>++</sup>] ( $P = 0.97$ ), and the velocity profiles (Fig. 1B3–1B6) are virtually identical to that in 5.5 mM Ca<sup>++</sup> (Fig. 1B1). Strikingly, at [Ca<sup>++</sup>] ≤ 10<sup>-5</sup> M, the aggregation effect is rapidly reversed and spontaneous pigment dispersion ensues, attaining 80%–100% of initial dispersion (Fig. 1A4–1A6) in the presence of RPCH. The mean dispersion velocity of about 5 μm/min is unaffected by [Ca<sup>++</sup>] ( $P = 0.67$ ). Subsequent perfusion with 5.5 mM Ca<sup>++</sup> induces full pigment aggregation (Fig. 1A4–1A6), and the translocation velocity profiles (Fig. 1B4–1B6) and peak velocities ( $P = 0.99$ ) are the same as in preparations perfused directly with 5.5 mM Ca<sup>++</sup> (Fig. 1B1).

Although the response to RPCH washout is immediate, dispersion is incomplete, reaching a maximum of about 50% (Fig. 1A2–1A6).

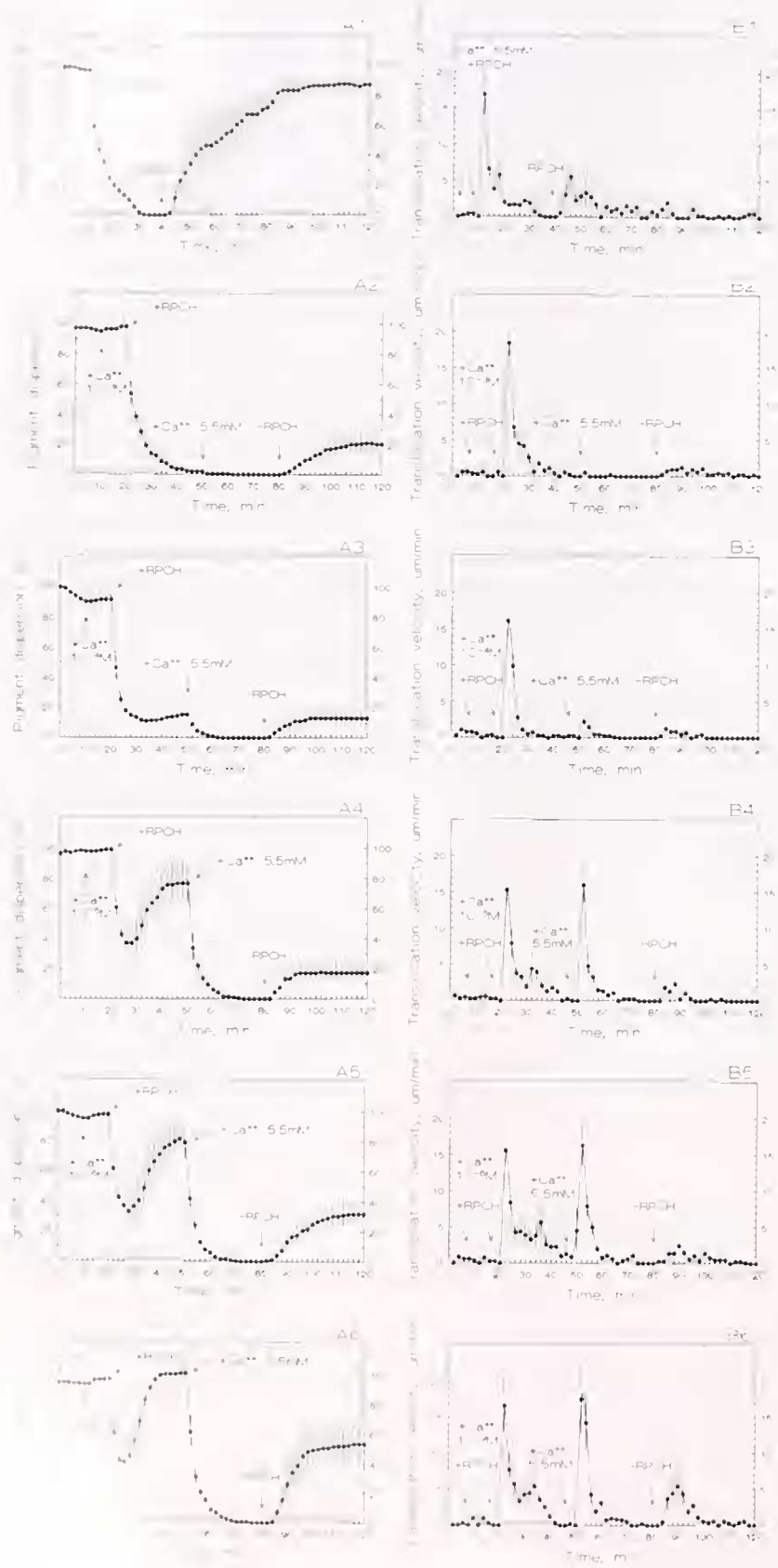
**Calcium channel blockers.** The time course and degree of pigment aggregation in response to 30 nM RPCH are unaffected by a 30-min preincubation with 5 mM Mn<sup>++</sup> (Fig. 2A) or 50 μM verapamil (Fig. 3A). The velocity profiles are also typical (Figs. 2B and 3B, respectively, *cf.* Fig. 1B1) and the maximum velocities (≈20 μm/min) are similar ( $P = 0.13$ ).

**Calcium ionophore A23187.** Perfusion with control (5.5 mM Ca<sup>++</sup>) saline causes no net movement of the dispersed pigment mass (initial 10 min of Fig. 4A1–4), although 10-min perfusion with the EGTA-buffered salines containing either 10<sup>-3</sup> (Fig. 4A2), 10<sup>-4</sup> (Fig. 4A3), or 10<sup>-5</sup> M Ca<sup>++</sup> (Fig. 4A4) induces progressive pigment aggregation ( $P < 0.001$ ) that attains a maximum of about 25% in 10<sup>-5</sup> M Ca<sup>++</sup> (Fig. 4A4). Translocation velocities are slow and range around a maximum of ≈2–4 μm/min (Fig. 4B2–4).

Perfusion with 25 μM A23187 while holding external Ca<sup>++</sup> at the designated reduced levels leads to complete aggregation within 26 min with 5.5 mM Ca<sup>++</sup> (Fig. 4A1) and virtually complete aggregation (90%) at 10<sup>-3</sup> M (Fig. 4A2). Peak aggregation velocities (11.6 ± 1.2 μm/min,  $n = 28$  [Fig. 4B1] and 9.0 ± 3.7 μm/min,  $n = 7$  [Fig. 4B2] respectively) are similar ( $P = 0.39$ ). However, with 10<sup>-4</sup> M Ca<sup>++</sup>, the initial aggregation response (7.4 ± 1.3 μm/min,  $n = 7$  [Figs. 4A3, 4B3]) reverses after about 8 min, and slow, steady (≈1 μm/min) pigment dispersion ensues (Fig. 4A3, 4B3). With 10<sup>-5</sup> M Ca<sup>++</sup>, A23187 induces slight, slow (≈1.3 μm/min) pigment dispersion (Fig. 4A4, 4B4).

Further perfusion with 5.5 mM Ca<sup>++</sup> produces complete, rapid pigment aggregation (Fig. 4A3, 4A4), regardless of the previous [Ca<sup>++</sup>] used. Velocity profiles and peak velocities (≈11 μm/min, Fig. 4B3, 4B4) do not differ ( $P = 0.83$ ) from those in preparations perfused directly with 5.5 mM Ca<sup>++</sup> (Fig. 4B1).

Removal of Ca<sup>++</sup> (≈7 × 10<sup>-11</sup> M Ca<sup>++</sup>, 2 mM EDTA) and A23187 from the perfusate after previous buffering



with Mg<sup>++</sup>-EGTA (Fig. 4A2–4) does not produce the customary full pigment dispersion seen with EDTA alone (cf. Fig. 4A1), possibly due to chelating interference by EGTA.

#### Dependence of pigment dispersion on reduced calcium

After complete pigment aggregation using 25  $\mu$ M A23187 and saline containing 5.5 mM Ca<sup>++</sup>, moderately rapid (maximum of  $6.8 \pm 1.7 \mu\text{m}/\text{min}$  [Fig. 5B1]), complete, pigment dispersion can be induced using a Ca<sup>++</sup>-free saline (residual Ca<sup>++</sup>  $\approx 7 \times 10^{-11}$  M, 2 mM EDTA [minutes 90–130 of Fig. 5A1]); removal of the ionophore itself does not induce dispersion.

As the [Ca<sup>++</sup>] in the Mg<sup>++</sup>-EGTA-buffered perfusate is decreased from  $10^{-7}$  (Fig. 5A2) to  $10^{-8}$  (Fig. 5A3) and  $10^{-9}$  M (Fig. 5A4), differential degrees of maximum pigment dispersion are induced, attaining about 80% in the latter. Maximum dispersion velocities increase ( $0.6 \pm 0.3$ ,  $2.8 \pm 2.1$ , and  $4.7 \pm 1.7 \mu\text{m}/\text{min}$ , respectively, [ $P = 0.03$ ]) with decreasing [Ca<sup>++</sup>] (Fig. 5B2–4). Further slight dispersion to about 90% can be induced using EDTA-buffered Ca<sup>++</sup>-free saline (minutes 90–130 of Fig. 5A4).

#### Relationship between degree of aggregation or dispersion and translocation velocity

Both translocation velocity and the degree to which pigment aggregates respond in a positively correlated, concentration-dependent manner to increasing external free [Ca<sup>++</sup>] between  $10^{-5}$  M and 5.5 mM (Fig. 6); half-maximum translocation velocity ( $V_{50}$ ) is reached at  $4.8 \times 10^{-5}$  M and half-maximum aggregation at  $3.2 \times 10^{-5}$  M external free Ca<sup>++</sup>. The velocity and degree of pigment dispersion are also positively correlated and increase in a concentration-dependent manner to decreasing external free [Ca<sup>++</sup>] between  $10^{-7}$  and  $10^{-11}$  M (Fig. 7), with  $V_{50}$  occurring at  $2.9 \times 10^{-9}$  M and half-maximum dispersion at  $7.6 \times 10^{-9}$  M external free Ca<sup>++</sup>.

### Discussion

The present data obtained with red pigment concentrating hormone (RPCH) and A23187 respectively reveal an extracellular and an intracellular calcium requirement for pig-

ment aggregation in the red ovarian chromatophores of *Macrobrachium olfersii*.

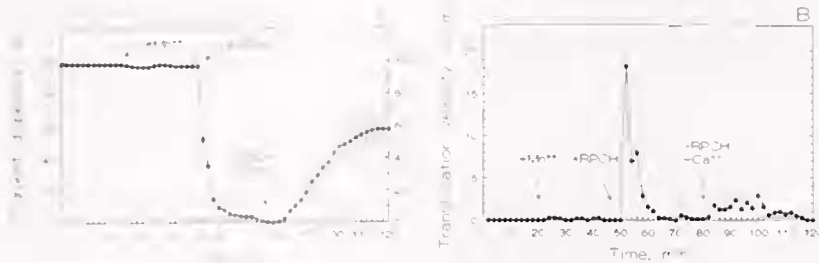
RPCH requires extracellular Ca<sup>++</sup> at  $\geq 10^{-4}$  M to induce complete pigment aggregation. At lower concentrations ( $10^{-5}$  to  $10^{-9}$  M Ca<sup>++</sup>), aggregation is incomplete ( $\approx 65\%$ ), although the maximum translocation velocities ( $\approx 16.5 \mu\text{m}/\text{min}$ ) and triphasic velocity profiles (McNamara and Ribeiro, 1999) are unaffected, except for minor suppression of the final low-velocity phase. This suggests that Ca<sup>++</sup> is required for coupling between RPCH and its receptor, much as in neural nicotinic receptors (Ospina *et al.*, 1998; Booker *et al.*, 1998; Liu and Berg, 1999). Incomplete aggregation may result from diminished signal transduction consequent to a reduction in the affinity between RPCH and its receptor at low [Ca<sup>++</sup>]. However, the intracellular signal generated is sufficient to transiently trigger the molecular motors responsible for aggregation at full capacity until the Ca<sup>++</sup>-regulatory mechanisms restore intracellular free Ca<sup>++</sup> to the resting concentration, leading to spontaneous dispersion.

Alternatively, an RPCH-activated, Ca<sup>++</sup>-induced/Ca<sup>++</sup>-release mechanism dependent on the Ca<sup>++</sup> gradient across the cell membrane (Putney and Bird, 1993; Verkhatsky and Shmigol, 1996) may operate. The reduced Ca<sup>++</sup> entering the chromatophore through RPCH-activated Ca<sup>++</sup> channels would be insufficient to promote sustained opening of target smooth endoplasmic reticulum Ca<sup>++</sup> channels, or to activate protein kinase C-dependent (Abrão *et al.*, 1991; Sugden and Rowe, 1992) or Ca<sup>++</sup>-calmodulin-dependent effector pathways (Lee *et al.*, 1994; Verkhatsky and Shmigol, 1996; Mukhopadhyay *et al.*, 1997), also resulting in transient pigment aggregation. Since the Ca<sup>++</sup>-channel blockers Mn<sup>++</sup> and verapamil have no effect on the degree and velocity of pigment aggregation, these putative receptor-activated Ca<sup>++</sup> channels seem not to be L-type channels (Britto *et al.*, 1996; Katz, 1996; Zhang *et al.*, 1997; Striessnig *et al.*, 1998). Although only a single maximum blocker dose was employed, and other Ca<sup>++</sup> channel types may be present, this suggests that the Ca<sup>++</sup> required to activate the granule transport motors may be intracellular rather than extracellular in origin.

When the limiting effect of low extracellular Ca<sup>++</sup> is bypassed by dissipating the Ca<sup>++</sup> gradient across the cell

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**Figure 1.** Dependence of RPCH-induced pigment aggregation (A series) on extracellular free Ca<sup>++</sup> (A1, 5.5 mM; A2,  $10^{-3}$  M; A3,  $10^{-4}$  M; A4,  $10^{-5}$  M; A5,  $10^{-6}$  M; A6,  $10^{-9}$  M) in *Macrobrachium olfersii* red chromatophores perfused sequentially with control (5.5 mM Ca<sup>++</sup>) saline, respective Ca<sup>++</sup>-10 mM EGTA-buffered saline, and Ca<sup>++</sup>-EGTA-buffered saline + 10 nM RPCH. At and below  $10^{-5}$  M Ca<sup>++</sup> (A4–A6), aggregation is only partial and followed by spontaneous dispersion. Peak RPCH-induced aggregation velocities (B series) are unaffected by external [Ca<sup>++</sup>] (B1, 5.5 mM; B2,  $10^{-3}$  M; B3,  $10^{-4}$  M; B4,  $10^{-5}$  M; B5,  $10^{-6}$  M; B6,  $10^{-9}$  M). RPCH washout with control saline leads to complete pigment dispersion (A1). Data are the mean  $\pm$  SEM ( $n = 7$ ).

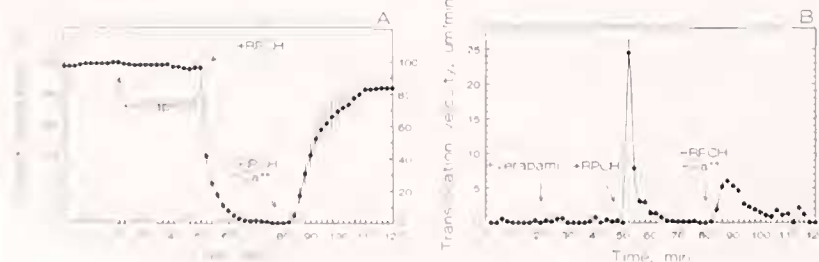


**Figure 2.** The calcium channel blocker  $Mn^{2+}$  has no effect on 30 nM RPCH-induced pigment aggregation (A) in *Macrobrachium olfersii* red chromatosomes perfused with control (5.5 mM  $Ca^{2+}$ ) saline and 5 mM  $MnCl_2$  for 30 min (cf., Fig. 1A1). The peak aggregation velocity (B) is normal (cf., Fig. 1B1), although dispersion during RPCH washout in  $Ca^{2+}$ -free saline is incomplete (A). Data are the mean  $\pm$  SEM ( $n = 7$ ).

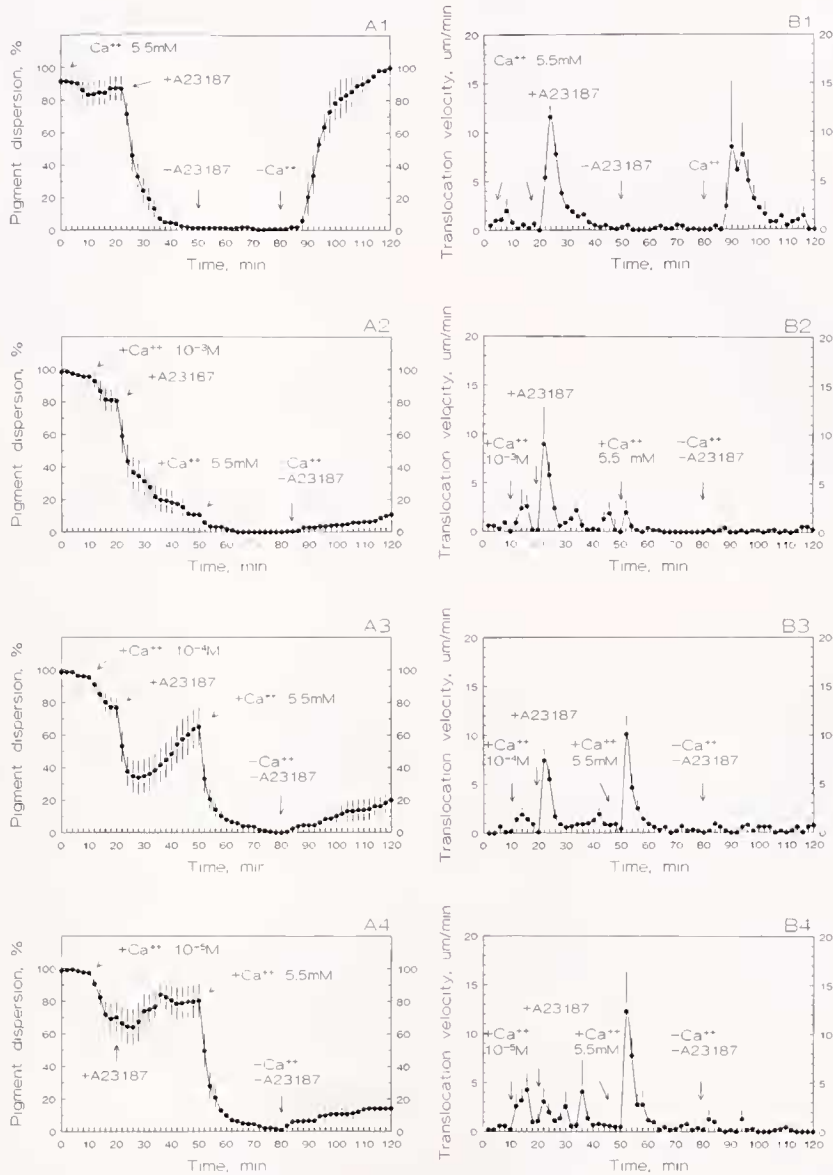
membrane with A23187, a second regulatory effect of  $Ca^{2+}$  appears. Assuming rapid equilibration of cytosolic  $Ca^{2+}$  with that in the extracellular medium (Lambert and Fingerman, 1978, 1979), both the degree of aggregation and the translocation velocity show  $Ca^{2+}$ -dependence below about  $10^{-3}$  M (Fig. 6). The transient aggregation followed by dispersion at  $10^{-4}$  M  $Ca^{2+}$  may result from decreased intracellular free  $[Ca^{2+}]$  consequent to the action of smooth endoplasmic reticulum (Treiman *et al.*, 1998) and plasma membrane  $Ca^{2+}$ -dependent ATPases (Carafoli, 1991). The mitochondrial electrogenic  $Ca^{2+}$  uniporter and  $H^+$  ( $Na^+$ )/ $Ca^{2+}$  antiporter (Pozzan *et al.*, 1994), and plasma membrane  $Na^+$ / $Ca^{2+}$  antiporter (Missiaen *et al.*, 1991; Thastrup, 1990) may also act to reduce intracellular free  $[Ca^{2+}]$ . At still lower concentrations ( $\leq 10^{-5}$  to  $\approx 10^{-11}$  M  $Ca^{2+}$ ), pigment dispersion ensues (Fig. 7). Thus, the direction of pigment movement, the translocation velocity of the edges of the pigment mass, and the degree of dispersion or aggregation appear to be calcium-dependent properties of the intracellular effector, the pigment translocation apparatus, or both.

Although the translocation mechanisms may depend on

very different pathways of signal transduction and activation and on diverse mechanochemical motor proteins, the present data corroborate estimates of the threshold  $[Ca^{2+}]$  necessary for pigment movements in teleost chromatophores; no such data are available for crustacean chromatophores. Pigment granules remain dispersed in cultured *Xiphophorus maculatus* erythrophores perfused with 10  $\mu$ M A23187 and EGTA-buffered,  $10^{-8}$  M free  $Ca^{2+}$ ; at  $10^{-4}$  M  $Ca^{2+}$ , erythroosome aggregation is almost complete (Oshima *et al.*, 1988). In isolated *Holocentrus ascensionis* erythrophores incubated in 10  $\mu$ M A23187 and EGTA-buffered, external free  $Ca^{2+}$  below  $5 \times 10^{-6}$  M, pigment granules are also dispersed; above  $5 \times 10^{-6}$  M  $Ca^{2+}$ , reversible aggregation is induced (Luby-Phelps and Porter, 1982). Microinjection of 10  $\mu$ M  $Ca^{2+}$  also induces erythroosome aggregation (Kotz and McNiven, 1994). In cultured *H. ascensionis* erythrophores, stripped of their plasma membranes with Brij 58 detergent to reveal the cytoskeleton, pigment granules aggregate rapidly at  $10^{-7}$  M free  $Ca^{2+}$  in an EDTA-EGTA buffer system; with  $10^{-8}$  M  $Ca^{2+}$  the granules disperse (McNiven and Ward, 1988). Direct measurements using Fura-2 show that intracellular  $[Ca^{2+}]$  in-



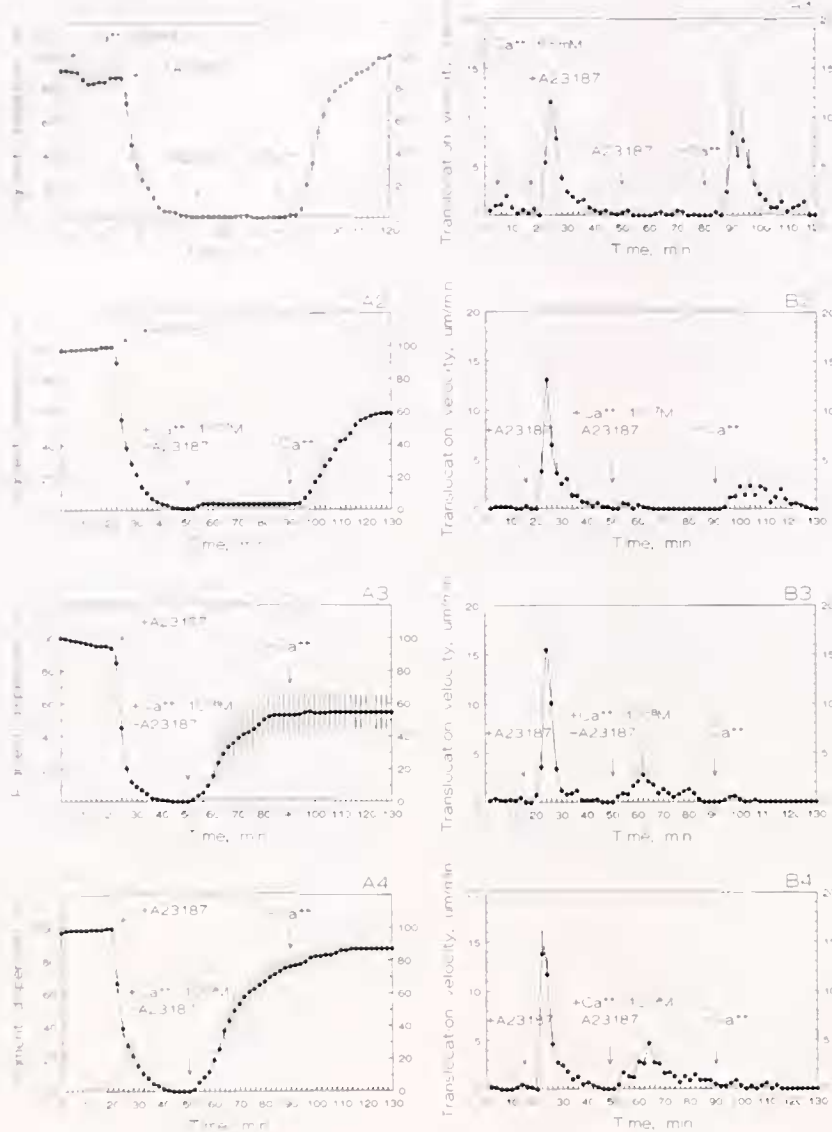
**Figure 3.** L-type calcium channel blocker verapamil has no effect on 30 nM RPCH-induced pigment aggregation (A) in *Macrobrachium olfersii* red chromatosomes perfused with control (5.5 mM  $Ca^{2+}$ ) saline and 5 mM  $MnCl_2$  for 30 min. Peak aggregation velocity (B) is normal (cf., Fig. 1B1), and dispersion during RPCH washout in  $Ca^{2+}$ -free saline is nearly complete (A). Data are the mean  $\pm$  SEM ( $n = 7$ ).



**Figure 4.** Dependence of ionophore-induced pigment aggregation (A series) on extracellular free Ca<sup>++</sup> (A1, 5.5 mM; A2, 10<sup>-3</sup> M; A3, 10<sup>-4</sup> M; A4, 10<sup>-5</sup> M) in *Macrobrachium olfersii* red chromatosomes perfused sequentially with control (5.5 mM Ca<sup>++</sup>) saline, respective Ca<sup>++</sup>-10 mM EGTA-buffered saline, and Ca<sup>++</sup>-10 mM EGTA-buffered saline + 25 μM A23187. At 10<sup>-4</sup> M external free Ca<sup>++</sup> (A3), pigment aggregation is only partial and followed by spontaneous dispersion. At 10<sup>-5</sup> M Ca<sup>++</sup>, pigment does not aggregate. The peak ionophore-induced aggregation velocities (B series) increase with increasing external [Ca<sup>++</sup>] (B1, 5.5 mM; B2, 10<sup>-3</sup> M; B3, 10<sup>-4</sup> M; B4, 10<sup>-5</sup> M). The slight aggregating effect in reduced [Ca<sup>++</sup>] prior to A23187 perfusion may reflect DMSO-induced efflux of intracellular Ca<sup>++</sup>. Data are the mean ± SEM (n = 7).

creases up to 100-fold or more, from about 30 nM in resting erythrofores to about 900 nM in epinephrine-stimulated *H. ascensionis* erythrofores (Kotz and McNiven, 1994). The present data, showing half-maximum aggregation at  $3.2 \times 10^{-5}$  M free Ca<sup>++</sup> and half-maximum dispersion at  $7.6 \times 10^{-9}$  M free Ca<sup>++</sup>, are consistent with these findings.

Although threshold intracellular Ca<sup>++</sup> concentrations for chromatophore pigment aggregation and dispersion remain to be estimated in nearly all crustacean species, pigment translocation in caridean and brachyuran chromatophores is clearly affected by if not dependent on alterations in intracellular [Ca<sup>++</sup>]. Lambert and Fingerman (1978) demon-



**Figure 5.** Dependence of pigment dispersion (A series) on decreasing external free  $\text{Ca}^{++}$  (A1, 5.5 mM; A2,  $10^{-7}$  M, A3,  $10^{-8}$  M, A4,  $10^{-9}$  M) in *Macrobrachium olfersii* red chromatosomes perfused sequentially with control (5.5 mM  $\text{Ca}^{++}$ ) saline, control saline + 25  $\mu\text{M}$  A23187, respective  $\text{Ca}^{++}$ -10 mM EGTA-buffered saline, and  $\text{Ca}^{++}$ -free saline. Pigment disperses only at extracellular free  $[\text{Ca}^{++}]$  below  $10^{-7}$  M (cf. A2 and A3). The peak dispersion velocities (B series) increase with decreasing external  $[\text{Ca}^{++}]$  (B1, 5.5 mM; B2,  $10^{-7}$  M; B3,  $10^{-8}$  M, B4,  $10^{-9}$  M). Data are the mean  $\pm$  SEM ( $n = 7$ ).

strated A23187-induced (25  $\mu\text{M}$ ) aggregation in *Palaemonetes pugio* ovarian chromatosomes perfused with Van der Meulen's saline (11 mM  $\text{Ca}^{++}$ ); subsequent perfusion with 10 mM EGTA saline results in intermediate aggregation. Native pigment dispersing in the chromatosomes induces granule dispersion in the chromatosomes of the epidermal chromatosomes of *Uca pugnax* in physiological saline (12.7 mM  $\text{Ca}^{++}$ ) (Quackenbush, 1981).

Curiously, 10 and 25  $\mu\text{M}$  A23187 not only inhibit the late phase (90–200 min) of PDH-induced pigment dispersion in *Uca pugnax* red chromatosomes, but also induce pigment aggregation (Quackenbush, 1981), much as in caridean chromatosomes. Complete aggregation in the polychromatic epidermal chromatosomes of *Palaemon affinis* is induced by an eyestalk extract delivered in physiological saline containing 10 mM  $\text{Ca}^{++}$ ; in  $\text{Ca}^{++}$ -free saline, pigment aggregation is only partial ( $\approx 30\%$ ; McNamara and

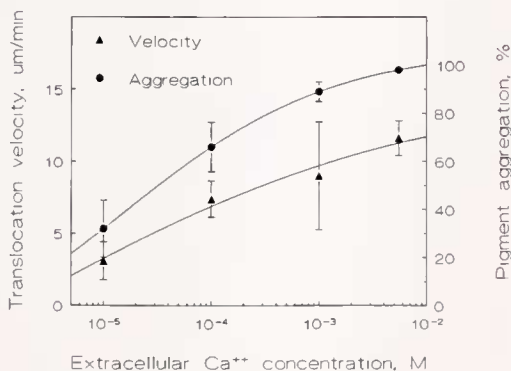


Taylor, 1987). In the red integumental chromatosomes of *Macrobrachium potiuma* perfused with 6.5 mM Ca<sup>++</sup>, A23187 (10<sup>-7</sup> to 10<sup>-4</sup> M) has a concentration-dependent aggregating effect, reversible by 10<sup>-8</sup> M  $\alpha$ -PDH (Britto *et al.*, 1990).

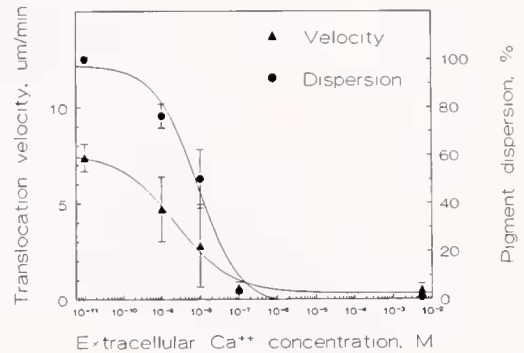
These various data demonstrate that pigment aggregation in the caridean shrimps and pigment dispersion in the brachyuran crabs can be brought about by changes in intracellular Ca<sup>++</sup> concentrations. The effector pathways of such concentration changes, whether increased by signal transduction or after A23187, are poorly known. Based on rightward shifting of the PCH dose-response curve by inhibitors of intracellular effectors, Nery *et al.* (1997) have suggested that pigment aggregation in *M. potiuma* red integumental chromatosomes may involve an inositol-trisphosphate-like cascade coupled to protein phosphatase 1 activation. This latter effect may down-regulate effector protein phosphorylation via the cyclic AMP-protein kinase A signaling pathway (Nery and Castrucci, 1997), given that increased intracellular cyclic AMP also induces pigment dispersion in caridean shrimps (Fingerman, 1969; Lambert and Fingerman, 1978; Nery *et al.*, 1998). Clearly, possible convergence or cross-talk between the Ca<sup>++</sup>-signaling and cyclic nucleotide-signaling pathways in crustacean pigment translocation mechanisms requires further investigation.

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**Figure 6.** Mean ( $\pm$ SEM,  $n \geq 7$ ) maximum pigment aggregation velocities ( $R^2 = 0.98$ ) and degree of maximum aggregation increase as a concentration-effect function ( $R^2 = 1.0$ ) of increasing extracellular free [Ca<sup>++</sup>] and are positively correlated (Pearson correlation,  $R = 0.98$ ,  $P = 0.02$ ). Data from *Macrobrachium olfersii* red chromatosomes perfused with respective Ca<sup>++</sup>-10 mM EGTA buffer and 25  $\mu$ M A23187.



**Figure 7.** Mean ( $\pm$ SEM,  $n \geq 7$ ) maximum pigment dispersion velocities ( $R^2 = 0.99$ ) and degree of maximum dispersion ( $R^2 = 0.98$ ) increase as a concentration-effect function of decreasing extracellular [Ca<sup>++</sup>] and are positively correlated (Pearson correlation,  $R = 0.98$ ,  $P = 0.003$ ). Data from *Macrobrachium olfersii* red chromatosomes perfused with respective Ca<sup>++</sup>-10 mM EGTA buffer after Ca<sup>++</sup> loading with 25  $\mu$ M A23187 and 5.5 mM Ca<sup>++</sup> for 30 min.

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